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AUTHOR(S):

Murata, Koichi; Yoshitomi, Hiroyuki; Furu, Moritoshi; Ishikawa, Masahiro; Shibuya, Hideyuki; Ito, Hiromu; Matsuda, Shuichi

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miR-451 Downregulates Neutrophil Chemotaxis via p38 Mitogen-Activated Protein Kinase

Koichi Murata[1], M.D.,PhD., Hiroyuki Yoshitomi[1,2], M.D.,PhD., Moritoshi Furu[1, 3], M.D.,PhD., Masahiro Ishikawa[1],M.D.,PhD., Hideyuki Shibuya[1], M.D., Hiromu Ito[1,3],M.D.,PhD. and Shuichi Matsuda[1]M.D.,PhD.

[1]Department of Orthopaedic Surgery, Kyoto University Graduate School of Medicine, Kyoto, Japan

[2]Center for Innovation in Immunoregulative Technology and Therapeutics, Kyoto University Graduate School of Medicine, Kyoto, Japan

[3]Department of the Control for Rheumatic Diseases, Kyoto University Graduate School of Medicine, Kyoto, Japan

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E-mail:

Address correspondence and reprint requests to:

Hiroyuki Yoshitomi, M.D., Ph.D.

Center for Innovation in Immunoregulative Technology and Therapeutics

Kyoto University Graduate School of Medicine

Yoshida Konoe-cho, Sakyo, Kyoto 606-8507, Japan

TEL +81-75-753-9501; FAX +81-75-753-9500

E-mail: yositomi@kuhp.kyoto-u.ac.jp

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Objective. MicroRNAs (miRNAs) are endogenous small noncoding RNAs that regulate the activities of target mRNAs and cellular processes. miR-451 is one of miRNAs conserved perfectly among vertebrates and regulates cell proliferation, invasion, and apoptosis in tumor. However, the role of miR-451 in autoimmune arthritis has been unknown. Our study was designed to identify the role of miR-451 in autoimmune arthritis.

Methods. We compared the expression of miR-451 in neutrophils from patients with rheumatoid arthritis (RA) and healthy controls (HCs). The role of miR-451 in neutrophil chemotaxis was evaluated in vivo and in vitro using neutrophils of mice. The regulation of p38 mitogen-activated protein kinase by miR-451 was assessed. Arthritis score and histology in SKG mice were examined by the administration of double-stranded miR-451.

Results. miR-451 expression was lower in neutrophils isolated from patients with RA than in those from HCs. Systemic administration of miR-451 significantly disturbed the infiltration of neutrophils in air pouch model without affecting apoptosis of neutrophils. Overexpression of miR-451 significantly suppressed the migration of neutrophils to formyl-methionyl-leucyl-phenylalanine. We identified CPNE3 and Rab5a as direct targets of miR-451. Overexpression of miR-451 suppressed the phosphorylation of p38 mitogen-activated protein kinase (MAPK) via 14-3-3ζ, a known target of miR-451, and Rab5a. In SKG mice, miR-451 treatment reduced the severity of arthritis and the number of infiltrating cells.

Conclusions. These results suggest that miR-451 suppresses neutrophil chemotaxis via p38 MAPK and that miR-451 is a potential therapeutic target in the treatment of RA.

Rheumatoid arthritis (RA) is a systemic, chronic inflammatory disease characterized by synovial hyperplasia, joint destruction, and extra-articular manifestations, and has a significant impact on both morbidity and mortality (1). Although the number of effective medications for RA has expanded rapidly, the pathogenesis of RA, in particular the role of microRNAs (miRNAs), remains to be determined.

miRNAs are endogenous small (~22 nucleotides) single-stranded, noncoding RNAs that mediate mRNA cleavage, translational repression, and mRNA destabilization (2); currently more than 2200 human miRNAs are registered (miRBase Release 19) (3). As fine-tuning regulators of gene expression, miRNAs have been implicated in important cellular processes such as apoptosis and differentiation (4), and it has been estimated that one-third of all mRNAs may be regulated by miRNAs (5).

Research has shown in the past several years that patients with RA have alterations in their cellular miRNAs. Dysregulation of miRNAs in PBMCs, T lymphocytes, synovial fibroblasts, and osteoclasts, each considered a key effector of joint destruction, has been shown to contribute to inflammation, degradation of the extracellular matrix, and invasive behavior of resident cells (6-9). miRNAs are also present in human plasma (called circulating miRNAs) (10). Altered expression of circulating miRNAs in patients with RA has been reported by our group and by others (11-13).

miR-451 is one of the miRNAs conserved perfectly among vertebrates and expressed abundantly in plasma (14,15). miR-451 plays an important role as a tumor suppressor by regulating cell proliferation, invasion, and apoptosis (16,17). miR-451 also

regulates cytokine production by dendritic cells (18). However, the role of miR-451 in autoimmune arthritis is unknown.

In the present study, we show that the cellular miR-451 level in neutrophils was lower in patients with RA than in healthy controls (HCs). We also demonstrate that enhancement of miR-451 suppressed neutrophil chemotaxis via downregulating the phosphorylation of p38 mitogen-activated protein kinase (MAPK) and that systemic administration of miR-451 with atelocollagen significantly suppressed neutrophil migration in air pouch model and severity of arthritis in SKG mice. These findings suggest that miR-451 has potential as a therapeutic target in the treatment of RA.

MATERIALS AND METHODS

Mice. SKG/Jcl mice and BALB/cCrSlc mice were purchased from Clea Japan (Tokyo, Japan) and Japan SLC (Shizuoka, Japan), respectively. All mice were maintained in a specific pathogen-free condition, and all animal studies were conducted in accordance with the principles of the Kyoto University Committee of Animal Resources, which are based on the International Guiding Principles for Biomedical Research Involving Animals.

Reagents. Mannan from *Saccharomyces cerevisiae* was purchased from Sigma-Aldrich (Tokyo, Japan) and was dissolved in 200 μl of Phosphate buffered saline (PBS) before i.p. injection. Formyl-methionyl-leucyl-phenylalanine (fMLP) and lipopolysaccharide (LPS) was obtained from Sigma-Aldrich. Human recombinant tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), interferon-γ (IFN-γ), and granulocyte macrophage colony-stimulating factor (GM-CSF) were purchased from PeproTech (Rocky Hill, NJ).

For flow cytometry, anti-mouse Ly-6G/Ly-6C (Gr-1) and anti-mouse CD11b antibodies, allophycocyanin–Annexin V, and 7- Amino-ActinomycinD (7-AAD) Viability Staining Solution were purchased from BioLegend (San Diego, CA). Anti-phospho-p38 MAPK (Thr180/Tyr182) was purchased from Cell Signaling Technology (Boston, MA). For western blot analysis, anti-β-actin and anti-Rab5a were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), anti-CPNE3 from Atlas Antibodies (Stockholm, Sweden), and anti-p38 and anti-phospho-p38 from Cell Signaling Technology.

Isolation of platelets, mononuclear cells (MNCs), and neutrophils. For human samples, ethical approval for this study was granted by the ethics committee of Kyoto University Graduate School and Faculty of Medicine. Written informed consent was obtained from all study participants. RA was diagnosed according to the criteria of the American College of Rheumatology (19). Samples of HC were collected from volunteers who were not being treated for arthralgia, heart failure, renal failure, or autoimmune disease.

Blood was drawn by cardiac puncture using EDTA-2K tubes. Blood was centrifuged at 600 g for 3 min. Platelet-rich plasma was further centrifuged for 2 min at 400 g to pellet the contaminating red blood cell (RBC) and then centrifuged for 5 min at 1300 g to pellet the platelets. Contaminating RBCs were lysed with Red Blood Cell Lysis Buffer (Roche Applied Science, Mannheim, Germany).

Human peripheral blood mononuclear cells (PBMCs) and neutrophils were isolated by Ficoll density centrifugation using Lymphocyte Separation Solution (d = 1.077 and d = 1.119; Nacalai Tesque, Kyoto, Japan), a Ficoll-based reagent with densities of 1.077 g/mL and 1.119 g/mL.

For RNA analysis for the murine neutrophil, neutrophils were purified with MACS cell separation system (Miltenyl Biotec, Bergisch Gladbach, Germany) using anti-Gr-1 antibody according to manufacturer's protocol.

Isolation of fibroblast-like synoviocytes (FLSs). FLSs from patients with RA were prepared as described before (11).

Total RNA isolation from tissue, cell samples, and conditioned medium. From cellular samples, RNA was extracted from cell samples using a High Pure miRNA Isolation Kit (Roche Applied Science) or RealTime ready Cell Lysis Kit (Roche Applied Science). Tissue samples were snap frozen in liquid nitrogen, homogenized with TriPure Isolation Reagent (Roche Applied Science), incubated for 5 min at room temperature, mixed with one fifth volume of chloroform, shaken vigorously for 15 seconds, incubated for 3 min and centrifuged at 12,000 g for 15 min at 4°C. Then 300 µl of aqueous phase was applied to the High Pure miRNA Isolation Kit according to the manufacturer's protocol. Total RNA included in the conditioned medium was isolated as described before (11,13).

Quantitative real-time PCR (qRT-PCR) of mature miRNAs. Reverse transcription was performed using an NCode VILO miRNA cDNA Synthesis Kit (Life Technologies). qRT-PCR was performed using EXPRESS SYBR GreenER qPCR SuperMix (Life Technologies), qRT-PCR was carried out on an Applied Biosystems 7500 Thermocycler (Life Technologies) with standard plasmids generated as described before (11,13) or synthetic first-strand cDNAs with the anticipated sequences. The forward primers were designed as described (11). The primer sequences are as follows: for U6, 5'-GCGGATTGGAACGATACAGAGAAGA-3'; for sno202, 5'-

GGCGCTGTACTGACTTGATGAAAG-3'; for miR-451, 5'-CGGGAAACCGTTACCATTACTGAGTT-3'. The data were analyzed with SDS Relative Quantification Software version 2.06 (Life Technologies). The absolute concentration of miRNAs in each sample was calculated as described before (11).

qRT-PCR of mRNA. Reverse transcription was performed using a Transcriptor High Fidelity cDNA Synthesis Kit or Transcriptor Universal cDNA Master (Roche Applied Science). qRT-PCR was performed using FastStart Universal SYBR Green Master (Roche Applied Science) on an Applied Biosystems 7500 Thermocycler according to the manufacturer's protocol. The following primer sequences were used: *GAPDH*: forward, 5'-TCTCGCTCCTGGAAGATGGT-3'; reverse, 5'- GGAAGGTGAAGGTCGGAGTC-3'; *CPNE3*: forward, 5'-GTCAGACCCTTTATGTGTGTTGT-3'; reverse, 5'-CGCTCAACCTCATACCACTGT-3'; *Rab5a*: forward, 5'-AGACCCAACGGGCCAAATAC-3'; reverse, 5'-TGGCTGCTTGTGCTCCTCTGTAG-3' and *14-3-3ζ*: forward, 5'-GCCCGTAGGTCATCTTGGAG -3' and reverse, 5'- TGTGAAGCATTGGGGATCAA -3'.

Intravenous injection of double-stranded miR-451 or negative control. Double-stranded miR-451, Cy5-conjugated double-stranded miR-451, and the nonspecific negative control (sequences: siRNA control: sense, 5'-AUCCGCGCGAUAGUACGUAUU-3'; antisense, 5'-UACGUACUAUCGCGCGGAUUU-3'; miR-451: sense, 5'-AAACCGUUACCAUUACUGAGUU-3'; antisense, 5'-

UAGUAAUGGUAAUGGUUCUC-3') were synthesized by FASMAC Co. Ltd. (Atsugi, Japan). Equal volumes of atelocollagen (Koken, Tokyo, Japan) and miRNA (20 μg/25 μl) were combined and mixed by rotation at 4°C for 20 min. Mice were injected with atelocollagen mixed with double-stranded miRNA in the tail vein.

Air pouch model mice. The air pouch model of local inflammation was induced in BALB/cCrSlc mice according to the method of Chalaris et al (20). Briefly, mice were anesthetized with pentobarbital and subcutaneous dorsal pouches were created by injection of 6 ml of sterile air. After 4 days, the pouches were reinjected with 4 ml of air. On day 6, 1 ml of 1% carrageenan (Sigma-Aldrich) in sterile PBS was injected into the pouches. The animals were anesthetized and the pouches were washed with 3 ml PBS. The lavage fluid was immediately cooled on ice and then analyzed by flow cytometry.

Under-agarose assay. For the under-agarose assay, neutrophils were isolated from spleen using a discontinuous Percoll (GE Healthcare, Tokyo, Japan) gradient comprising a stock Percoll solution (90 ml Percoll, 10 ml 10× Hanks' balanced salt solution (HBSS)) diluted to 72%, 64%, and 52% in PBS as described (21). The neutrophil band was removed, then cells were washed in PBS, treated with Red Blood Cell Lysis Buffer to lyse the contaminating RBC, washed in PBS, and suspended in HBSS plus 10% murine plasma at 1.0×10^7 cells/ml.

The under-agarose assay was performed as described previously (21,22). Briefly 35 mm culture dishes were filled with 3 ml of a 1.2% agarose solution containing 50% H_2CO_3-buffered HBSS and 50% RPMI 1640 culture medium supplemented with 20% heat-inactivated fetal bovine serum. After the agarose solidified, three holes, 3.5 mm in diameter and 2.4 mm apart, were cut into a straight line in the gel. The gels were allowed

to equilibrate for 1 h in a 37°C/5% CO_2 incubator. The central well was loaded with 10 µl of purified neutrophils and the outer wells were loaded with chemoattractant. Gels were incubated 4 h in a 37°C/5% CO_2 incubator.

Flow cytometry. Neutrophils were stained for Gr-1, CD11b, Annexin V and 7-AAD, and analyzed on a BD FACSCanto II (BD Biosciences, San Jose, CA). To quantify p-p38 MAPK, neutrophils from spleens were stained for Gr-1, CD11b, and phospho-p38 MAPK according to the manufacturer's recommendation.

Plasmid construction. The miR-451 overexpression vector (pcDNA6.2/miR-451) was generated by ligating annealed oligonucleotides into pcDNA6.2-GW/EmGFP-miR vector (Life Technologies), using BLOCK-iT Pol II miR RNAi Expression Vector Kits (Life Technologies). A construct inserted with oligonucleotides synthesized in reference to the non-specific negative control of miCENTURY OX miNatural (Cosmo Bio, Tokyo, Japan) was used for the pcDNA6.2/mock, and oligonucleotides of anti-miR-451 sequences were used for pcDNA6.2/anti-miR-451. The sequences of inserted oligonucleotides are as follows: mock: sense, 5'-TGCTGAAATCCGCGCGATAGTACGTAGTTTTGGCCACTGACTGACTACGTACTCGCGCGGATTT-3'; antisense, 5'-CCTGAAATCCGCGCGAGTACGTAGTCAGTCAGTGGCCAAAACTACGTACTATCGCGCGGATTTC-3'; miR-451: sense, 5'-TGCTGAAACCGTTACCATTACTGAGTTGTTTTGGCCACTGACTGACAACTCAGTTGGTAACGGTTT-3'; antisense, 5'-CCTGAAACCGTTACCAACTGAGTTGTCAGTCAGTGGCCAAAACAACTCAGTAATGGTAACGGTTTC-3'; and anti-miR-451: sense, 5'-

TGCTGAACTCAGTAATGGTAACGGTTTTGGCCACTGACTGAACCGTTAATTAC TGAGTT-3'; antisense, 5'-GGCCAAAACCGTTACCATTACTGAGTTC-3'.

To construct the luciferase reporter vector, we inserted a 3′-untranslated region (3′UTR) fragment containing putative binding sites for miR-451 into the NheI-SalI fragment of the pmirGLO vector (Promega, Fitchburg, WI). The sequences of inserted oligonucleotides are as follows: CPNE3 3'UTR wild-type (wt): sense, 5'-CTAGCTAGCGGCCGCTAGTAATTGAGATTTGTTAAAACGGTTAG-3'; antisense, 5'- TCGACTAACCGTTTTAACAAATCTCAATTACTAGCGGCCGCTAG-3'; CPNE3 3'UTR mutant (mt): sense, 5'-CTAGCTAGCGGCCGCTAGTAATTGAGATTTGTTAAGGAAACCAG-3'; antisense, 5'- TCGACTGGTTTCCTTAACAAATCTCAATTACTAGCGGCCGCTAG-3'; Rab5a 3'UTR wt: sense, 5'-CTAGCTAGCGGCCGCTAGTAATGCAGAATTAGGAAAACGGTTCG-3'; antisense, 5'-TCGACGAACCGTTTTCCTAATTCTGCATTACTAGCGGCCGCTAG-3'; Rab5a 3'UTR mt: sense, 5'-CTAGCTAGCGGCCGCTAGTAATGCAGAATTACCAAATGCCTTCG-3'; antisense, 5'-TCGACGAAGGCATTTGGTAATTCTGCATTACTAGCGGCCGCTAG-3'.

Transfection. HeLa cells were seeded into 12-well, 24-well, or 96-well plates as appropriate and transfected with the precursor-miR-451-expressing vector or the mock vector with or without the luciferase reporter vector using FuGene HD (Roche Applied Science). Double-stranded miRNA (miCENTURY OX miNatural) and the non-specific negative control siRNA were purchased from Cosmo Bio. The siRNA sequences specific

for 14-3-3ζ, Rab5a, and CPNE3 have been described (23-25). Transfection of siRNA with or without the luciferase reporter vector was performed using X-tremeGENE siRNA Transfection Reagent (Roche Applied Science).

FLSs were seeded into 96-well plates as appropriate and transfected with double-stranded miRNA or siRNA using a TransIT-TKO transfection reagent (Mirus, Madison, WI).

Luciferase assay. Luciferase activity was measured 24 h after transfection using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instruction.

Western blot analysis. Western blot analysis was performed as described before (26). Briefly, 10–20 ng of cell lysate was subjected to 10% SDS-PAGE and transferred onto a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). After blocking with 1% skim milk or 2% BSA, the membrane was probed with anti-β-actin (1:4000), anti-Rab5a (1:1000), anti-CPNE3 (1:1000), anti-p38 (1:1000), or anti-phospho-p38 (1:1000) antibody, incubated with horse radish protein-conjugated second antibody, and visualized using Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, Rockford, IL), ECL Plus Western Blotting Detection Reagent (GE Healthcare UK, Buckinghamshire, UK), or ECL Prime Western Blotting Detection Reagent (GE Healthcare UK), as appropriate.

Enzyme-linked immunosorbent assay (ELISA). The quantification of p38 and phospho-p38 MAPK was performed using Cell-Based P38 MAPK (Thr180/Tyr182) ELISA (RayBiotech, Norcross, GA).

The concentrations of IL-6 in culture medium of FLSs were quantified with Human IL-6 ELISA MAX (BioLegend).

Clinical assessment of arthritis scores. The arthritis score was assessed as described previously (27).

Histological analysis. Ankle joint specimens from the mice were processed as 5-μm–thick-paraffin-embedded sections and stained with hematoxylin and eosin.

Statistical analysis. The in vivo joint scores were analyzed by the Mann-Whitney *U* test. Unless otherwise mentioned, the Student's *t* test was used for statistical analysis. $P < 0.05$ was considered significant.

RESULTS

miR-451 expression in neutrophils is significantly lower in RA patients. First we analyzed the expression of miR-451 in human blood cells. miR-451 was expressed the most abundantly in RBCs, followed by platelets and neutrophils (Figure 1A). Among them, miR-451 expression in neutrophils was significantly lower in RA patients than in HCs (Figure 1B). To examine whether proinflammatory signals induce downregulation of miR-451 in neutrophils, neutrophils were stimulated with LPS, TNF-α, IL-1β, IFN-γ, or GM-CSF. Although miR-451 expression was not changed by LPS, TNF-α or IL-1β stimulation, IFN-γ, and GM-CSF each induced the downregulation of miR-451 in neutrophils ($p < 0.05$, $p < 0.01$, respectively) (Figure 1C). Combination of IFN-γ and GM-CSF did not enhance the downregulation of miR-451. These data suggest that the expression of miR-451 in neutrophils of RA patients might be decreased in response to

cytokine stimuli and that the change in miR-451 has potential roles in neutrophil function.

miR-451 suppresses neutrophil chemotaxis. To investigate the relevance of miR-451 to the neutrophil function, we used the air pouch model, a reliable experimental approach for studying the inflammatory mechanisms such as neutrophil chemotaxis (28). After generation of the air pouch, double-stranded miR-451 or control RNA was administered intravenously to BALB/c mice, followed by local injection of carrageenan into the air pouch. Twenty-four hours later, the numbers of total cells and neutrophils in the air pouch were counted by flow cytometry (Figure 2A). First, we confirmed the effective in vivo transfection (~45%) of the Cy5-conjugated double-stranded miR-451 into Gr-1+CD11b+ neutrophils and that the expression of miR-451 in neutrophils from mice transfected with double-stranded miR-451 was 1.8 times as high as in neutrophils from control mice (Figures 2B and C).

Administration of miR-451 significantly decreased the infiltration by all cells (Figures 2D –F) and by Gr-1+CD11b+ neutrophils (Figure 2G). miR-451 reportedly induces apoptosis via Rab14 in human non-small cell lung cancer (17). However, apoptosis did not increase in the accumulated neutrophils of miR-451-treated mice (Figures 2H and I), indicating that apoptosis was not involved in the decreased infiltration by neutrophils.

There were no significant differences in the number of neutrophils in peripheral blood and the mean fluorescence intensity of Gr-1 in the accumulated neutrophils (data not shown).

To exclude the influence of the capsule cells of the air pouch, neutrophils obtained from miR-451-treated or control mice were conducted into under-agarose assay (21). Figure 2J shows that miR-451 overexpression decreased neutrophil migration toward fMLP.

These data indicate that overexpression of miR-451 in neutrophils decreases the ability of chemotaxis to the site of inflammation.

miR-451 directly targets CPNE3 and Rab5a genes. miR-451 is known to suppress Rab14 (17), 14-3-3ζ (29,30), and CUGBP2 (31) directly. To investigate the roles of miR-451 in neutrophils, we tried to find new target genes of miR-451. First, we generated a miR-451 expression vector, which upregulated miR-451 expression by 8- and 15-fold, 24 and 48 h after transfection, respectively (24 h: data not shown; 48 h: Figure 3A).

Computational screening for target genes having complementary sites for miR-451 in their 3′UTR using open access software including TargetScan (32) and miRanda (33), predicted more than 1600 putative target genes. We selected 12 candidate genes that were expected to be involved in neutrophil function, and we inserted the 3′UTR sequences of each candidate gene into the downstream of the firefly luciferase reporter gene of the pmirGLO vector. The pmirGLO vector and the miR-451-overexpressing vector, control vector, or anti-miR-451-overexpressing vector were co-transfected into HeLa cells, and luciferase activity was measured (Figures 3B–D).

Overexpression of miR-451 downregulated the activity of luciferase with the target sequences of CPNE3 and Rab5a in the downstream of the firefly luciferase gene, while suppression of miR-451 upregulated the luciferase activity (Figures 3C and D). Although,

luciferase with mutated 3′UTR sequences of Rab5a was paradoxically influenced by miR-451 vector, luciferase with mutated 3′UTR sequences of CPNE3 was not influenced by overexpression or downregulation of miR-451 as expected. Transfection of synthesized double-stranded miR-451 also decreased the activity of luciferase of each pmirGLO vector (Figure 3E). Overexpression of miR-451 also significantly downregulated the expression of native CPNE3 and Rab5a of HeLa cells at the protein and mRNA levels in the HeLa cells (Figures 3F and G). By contrast, downregulation of miR-451 increased these expression levels (Figures 3F and G). These data suggest that miR-451 affects the expression of CPNE3 and Rab5a at both the transcriptional and translational levels.

Expressions of CPNE3 and Rab5a in human neutrophils were shown previously (34,35). We also analyzed the expression of CPNE3 and Rab5a in neutrophils from patients with RA and HCs, and there were no significant differences in the expression between RA and HC (data not shown). However, the expression of Rab5a had a negative correlation with the expression of miR-451 in RA patients ($R = -0.83$, $p < 0.05$), indicating the regulation of Rab5a by miR-451 in neutrophils of RA patients (Figure 3H).

miR-451 downregulates the p38 MAPK signaling pathway via 14-3-3ζ, and Rab5a. During the host response to bacterial infection or tissue injury, neutrophils respond to multiple chemoattractants. However, the neutrophils must ultimately disengage from some chemoattractants and migrate unidirectionally toward the bacteria-derived chemoattractants (21). Neutrophils prioritize chemotactic signals by distinguishing ‘intermediary’ (LTB4, CXCL8, platelet-activating factor) and ‘end-target’ (fMLP, C5a) chemoattractants through distinct intracellular signaling pathways.

Although the phosphoinositide 3-kinase pathway is important in the intermediary chemoattractant pathway, p38 MAPK plays an important role in migration of neutrophils toward the end-target chemoattractant.

To evaluate the effect of miR-451 on p38 MAPK, we made siRNAs for Rab5a, CPNE3, and 14-3-3ζ because 14-3-3ζ is thought to associate with p38 MAPK (36). 14-3-3ζ, Rab5a, and CPNE3 mRNA were degraded when HeLa cells were transfected with siRNA for each gene (Figures 4A–C). Transfection of double-stranded miR-451 also significantly decreased 14-3-3ζ, Rab5a, and CPNE3 mRNA levels (Figures 4A–C).

Western blot analysis showed that the expression of p38 MAPK was not affected in cells transfected with double-stranded miR-451 or with siRNA for 14-3-3ζ, Rab5a, or CPNE3, whereas the phosphorylation of p38 MAPK was downregulated (Figure 4D). The amounts of p38 or phosphorylated p38 (p-p38) MAPK was quantified by ELISA (Figures 4E and F). p-p38 MAPK content decreased in cells transfected with double-stranded miR-451, 14-3-3ζ siRNA, or Rab5a siRNA. These data suggest that upregulation of miR-451 expression inhibited the phosphorylation of p38 MAPK via 14-3-3ζ and Rab5a but not CPNE3. No significant effect on the phosphorylation of Akt, a downstream molecule of the phosphoinositide 3-kinase pathway, was observed in cells transfected with double-stranded miR-451 (data not shown).

Next, we harvested neutrophils from the spleens of mice administered with double-stranded miR-451 or control siRNA intravenously. Flow cytometric analysis showed that miR-451 overexpression decreased p-p38 MAPK in Gr-1+CD11b+ neutrophils (Figures 4G and H). These data suggest that overexpression of miR-451 decreases p-p38 MAPK also in mice neutrophils.

To further determine a role of miR-451 and its target genes including Rab5a and CPNE3, we investigated IL-6 production from RA FLS. First, we confirmed that transfection of double-stranded miR-451 significantly decreased 14-3-3ζ, Rab5a, and CPNE3 mRNA levels, even in RA FLS (Figure 4I). While transfection of 14-3-3ζ siRNA enhanced the production of IL-6 ($p < 0.01$), overexpression of miR-451 and knockdown of Rab5a or CPNE3 downregulated the production of IL-6 ($p < 0.01$, < 0.05, and $p = 0.09$, respectively; Figure 4J), implying miR-451 is involved in the pathogenesis of RA including the priming of neutrophils via IL-6(37).

Administration of double-stranded miR-451 ameliorates autoimmune arthritis. As indicated above, we demonstrated that administration of miR-451 markedly decreased the infiltration of neutrophils in the air pouch model. However, various types of immune cells also play roles in the etiopathogenesis of RA. SKG mice, which harbor a mutation of the gene encoding ZAP-70 on a BALB/c background, develop self-sustained arthritis after a single injection of mannan (38). To examine the involvement of miR-451 in the development of autoimmune arthritis, we injected double-stranded miR-451 intravenously twice a week into mannan-treated SKG mice.

Intravenous administration of miR-451 reduced the severity of arthritis significantly (Figures 5A and B) and reduced the incidence slightly (Figure 5C). Histological analysis indicated that synovitis was suppressed and that the number of cells infiltrating the synovium was reduced significantly in mice treated with double-stranded miR-451 (Figures 5D and E). Attenuation of arthritis by administration of miR-451 suggests that downregulation of miR-451 expression might lead to exacerbation of autoimmune

arthritis partly by dysregulation of the chemotaxis of infiltrating cells and that administration of miR-451 is a potential treatment for RA.

Discussion

miRNAs are of interest because they play a crucial role as a fine regulator of gene expression at the post-transcriptional level within the cell in many diseases, and because miRNA is a potential therapeutic target. The abnormal expression pattern of miRNAs has been reported in various pathologies, and the suppression of overexpressed miRNAs or reconstitution of the expression by restoration of silenced miRNAs is a therapeutic target in many fields (4).

miR-451 has been shown to play an important role in erythropoiesis (39) and in suppressing tumor growth by regulating cell proliferation and apoptosis (16,17). A contribution of miR-451 to CD4+ T cell function has also been suggested (40). Thus, we assumed that miR-451 had relevance to autoimmune arthritis and reduced expression of miR-451 in neutrophils implies the possibility that miR-451 plays a crucial role in the neutrophil function.

Transcriptional regulation of miR-451 is largely unknown, however, miR-451 was shown to be transcriptionally activated by E2A in T cells (41). E2A activity is inhibited by RAS– extracellular signal-regulated kinase (ERK)–MAPK pathway (42), which is activated by GM-CSF stimulation (43). EGR1, an inhibitory factor for E2a, is stimulated by IFN-γ independently of STAT-1 (44). Stimulation with GM-CSF or IFN-γ might downregulate the expression of miR-451 in neutrophils via this pathway.

Neutrophil chemotaxis driven by the complement pathway plays a crucial role in animal models of arthritis (45), and the possible contribution of neutrophils even in the early phases of RA pathophysiology has been reported (46). Neutrophils are the first cell type to arrive at sites of inflammation. They secrete immune mediators that can activate neutrophils and other immune cells, triggering positive regulatory feedback that may lead to acute and persistent inflammation. Neutrophils live longer in inflammatory sites, where they augment the release of powerful destructive enzymes. These cells are also thought to be involved in bone remodeling and bone resorption through their membrane-bound RANKL, which activates monocyte fusion into fully functional osteoclasts (47). Neutrophils can adopt different phenotypes such as antigen presenting cell and osteoclast-like cells. Thus, neutrophils are considered to be more than simple final effectors.

Rab5a and CPNE3 are newly identified direct targets of miR-451. Rab5a is small GTPase and plays an important role in endosome formation and trafficking in neutrophils and macrophages (48,49). CPNE3 exists in the cytosol of neutrophils and is expressed in the immature neutrophil precursors (34). However, the true function of CPNE3 in neutrophils is unknown because the short lifespan of neutrophils limits the possibility of using transfection or in vivo microinjection studies.

Systemic administration of miR-451 suppressed the arthritis in SKG mice. Although the reduced chemotaxis of neutrophils by miR-451 explains at least part of this suppression, the contribution of other cells may also be assumed. Via Rab5a and CPNE3, miR-451 downregulated the production of IL-6 from FLS, which plays a fundamental role in driving the inflammation associated with RA at both the local and systemic levels.

We also confirmed that miR-451 downregulated the proliferation of FLS independent of Rab5a and CPNE3 (data not shown). A recent study demonstrated that inhibition of miR-451 in dendritic cells increased the production of IL-6, TNF, CCL3, CCL5, and IFN-β via 14-3-3ζ when cells were transfected with influenza (18). Proinflammatory mediators including TNFα, IL-1β and COX-2 lie downstream of p38 MAPK (50). Systemic administration of miR-451 might relieve inflammation in autoimmune arthritis via these mechanisms.

The present study has a few limitations. First, it is difficult to deliver pre-miR-451 specifically to neutrophils. We confirmed that the expression of miR-451 in neutrophils from mice transfected with double-stranded miR-451 was 1.8 times as high as in neutrophils from control mice. However, surrounding cells, which were also transfected with miR-451, might prime the condition of neutrophils in vivo prior to isolation. More efficient direct methods to deliver miRNA are needed for the clinical use of small RNAs.

Next, although we showed that miR-451 inhibits the phosphorylation of p38 MAPK via 14-3-3ζ and Rab5a, it remains to be determined how crucial these genes are for the regulation of p38 MAPK by miR-451.

Another limitation of this study is that we could not identify the function of CPNE3 in neutrophils, and we could not elucidate fully the mechanism by which miR-451 suppresses autoimmune arthritis. The function of CPNE3 in neutrophils and the role of miR-451 in other cells including T, B, and synovial cells should be investigated in future. Nevertheless, this study shows for the first time the obvious suppressive function of miR-451 in neutrophil chemotaxis and in autoimmune arthritis.

In conclusion, we demonstrated that miR-451 suppressed neutrophil chemotaxis via p38 MAPK in vitro and in vivo and ameliorated autoimmune arthritis. In the process of analysis we identified new direct targets of miR-451. These findings suggest that miR-451 is a potential therapeutic target in the treatment of RA.

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AUTHOR CONTRIBUTIONS

Dr. Yoshitomi had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Yoshitomi, Murata

Acquisition of data. Murata, Yoshitomi

Analysis and interpretation of data. Yoshitomi, Murata, Ito, Matsuda

Manuscript preparation. Yoshitomi, Murata

Statistical analysis. Murata, Yoshitomi

Discussion. Yoshitomi, Murata, Ito, Furu, Shibuya, Ishikawa, Matsuda

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Figure legends

Figure 1.

miR-451 expression was significantly reduced in neutrophils from patients with rheumatoid arthritis (RA). **A** and **B,** The expression of miR-451 normalized by the expression of U6 was quantified by quantitative real-time PCR (qRT-PCR). Data are shown as mean ± SD. The miR-451 expressions in blood cells from 14 healthy controls (HCs)(**A**) and in neutrophils from patients with RA (n = 7) and HCs (n = 10)(**B**). **C,** Human neutrophils were cultured for 18 h in medium alone or in the presence of LPS (30 ng/ml), TNF-α (2 ng/ml), IL-1β (2 ng/ml), IFN-γ (5 ng/ml), and/or GM-CSF (30 pM). The expression of miR-451 normalized by the expression of U6 in neutrophils was quantified by qRT-PCR (n = 4). Data are shown as mean ± SEM. * = $P < 0.05$; ** = $P < 0.01$.

Figure 2.

Overexpression of miR-451 decreased the chemotaxis ability. **A,** Scheme showing the air pouch model of local inflammation in BALB/c mice as outlined in Methods. Double-stranded miR-451 or control siRNA was injected into the tail vein 24 h before carrageenan injection. **B,** Cy5-positive or Cy5-negative miR-451 was administered intravenously with atelocollagen, and the uptake of miRNA by neutrophils in the peripheral blood was assessed by flow cytometry 24 h later. The percentage of Cy-5-positive cells is indicated. **C,** Neutrophils were obtained from spleens of mice that had been treated with double-stranded miR-451 or control siRNA 24 h before isolation. miR-451 expression normalized by the expression of sno202 in neutrophils was quantified by qRT-PCR (n = 8). **D,** The pellet obtained from the air pouch lavage fluid. **E**-**I**, Cells that had infiltrated into the lavage fluid were stained for Gr-1, CD11b, Annexin V, and 7-AAD, and counted by flow cytometry. Representative expression of Annexin V and 7-AAD on Gr-1+CD11b+ cells is shown in **E**. The numbers of total cells (**F**) and Gr-1+CD11b+ neutrophils (**G**) were counted. The number of Annexin $V^{-}7\text{-}AAD^{-}$ cells (**H**) and Annexin V+7-AAD+ cells (**I**) in Gr-1+CD11b+ cells were also shown (n = 8). **J,** Migration of neutrophils to gradients of the chemoattractant was evaluated by the under-agarose assay. Neutrophils were obtained from spleens of mice that had been treated with double-stranded miR-451 or control siRNA 24 h before isolation. Ten microliters of 0.1 μM of fMLP was loaded into the outer wells of the gel (n = 5). Data are shown as mean ± SEM. * = $P < 0.05$; n.s. = not significant.

Figure 3.

miR-451 directly targeted *CPNE3* and *Rab5a* genes. **A,** HeLa cells were transfected with pcDNA6.2/miR-mock (pc/mock), or pcDNA6.2/miR-451 (pc/miR-451) vector. The miR-451 expression normalized by U6 expression in HeLa cells was quantified by qRT-PCR 48 h after transfection (n = 6). **B,** Putative miR-451-binding sequence in the 3′-untranslated region (3′UTR) of CPNE3 and RAB5a mRNA. A human CPNE3 or Rab5a 3′UTR fragment containing wild type (wt) or mutant (mt) miR-451-binding sequence was inserted downstream of the *luciferase* reporter gene. Seed sequences complementary to the target sequences are indicated in bold. **C** and **D**, The wild type or mutant reporter plasmid containing 3′UTR fragment of CPNE3 (**C**) or Rab5a (**D**) was co-transfected into HeLa cells with the pc/mock, pc/miR-451, or pcDNA6.2/anti-miR-451 (pc/anti-miR-451) vector (n = 6). **E,** The wild type reporter plasmid containing 3′UTR fragment of CPNE3 or Rab5a was co-transfected into HeLa cells with double-stranded miR-451 (ds miR-451) or negative control siRNA (ds NC) (n = 3). **F** and **G**, HeLa cells were transfected with the pc/mock, pc/miR-451, or pc/anti-miR-451 vector. The expression of CPNE3 and Rab5a protein (F) was analyzed by western blot assay (Densitometric analysis data of n = 4 experiments). The expression of *CPNE3* and *Rab5a* mRNA relative to *GAPDH* (G) was analyzed by qRT-PCR assay (n = 6). **H,** The correlation between the expression of miR-451 and CPNE3 or Rab5a in neutrophils from patients with RA (n = 7) was analyzed. The relative expressions of miR-451, CPNE3 and Rab5a normalized U6 or *GAPDH* are shown. Data are shown as mean ± SEM. * = $P < 0.05$; ** = $P < 0.01$.

Figure 4.

Overexpression of miR-451 inactivated the p38 MAPK signaling pathway via 14-3-3ζ, and Rab5a. **A**−**C**, The expression levels of *14-3-3ζ* (**A**), *CPNE3* (**B**), and *Rab5a* (**C**) mRNA relative to *GAPDH* were analyzed by qRT-PCR assay in HeLa cells transfected with negative control siRNA, double-stranded miR-451, or siRNA for 14-3-3ζ, Rab5a or CPNE3 (n = 6, respectively). **D,** Western blots of whole-cell extracts from negative control siRNA, double-stranded miR-451, 14-3-3ζ siRNA, Rab5a siRNA, or CPNE3 siRNA-transfected HeLa cells. β-actin was used as a loading control. Representative images of three independent experiments are shown. **E** and **F**, The amounts of p38 MAPK (**E**) and phospho-p38 MAPK (p-p38) (**F**) were quantified by ELISA (n = 6, respectively). **G,** p-p38 MAPK in Gr-1+CD11b+ neutrophils was detected by flow cytometry. Neutrophils were harvested from spleens of mice 24 h after intravenous administration of double-stranded miR-451 or control siRNA. **H,** The percentage of p-p38+ cells and the mean fluorescence intensity (MFI) of p-p38 were quantified (n = 5, respectively). **I,** The expression levels of 14-3-3ζ, CPNE3, and Rab5a mRNA relative to GAPDH were analyzed by qRT-PCR assay in fibroblast-like synoviocytes (FLSs) 48 h after transfection with negative control siRNA, or double-stranded miR-451 (n = 4, respectively). **J,** After the transfection with negative control siRNA, double-stranded miR-451 or siRNAs for Rab5a, CPNE3, and 14-3-3ζ for 48 h, FLSs were cultured in serum-free DMEM with 10 ng/ml TNF-α for 48 h. The concentration of IL-6 in the conditioned medium was quantified with ELISA (n = 5). Data are shown as mean ± SEM. * = $P < 0.05$; ** = $P < 0.01$.

Figure 5.

Administration of double-stranded miR-451 ameliorated autoimmune arthritis in SKG mice. **A**−**C**, 8–12-week-old female SKG mice received a single i.p. injection of 20 mg of mannan. Double-stranded miR-451 or control siRNA was administered intravenously on days 0, 4, 7, and 11. Representative joint swelling (**A**), graphical summary of arthritis scores (**B**), and incidence of arthritis (**C**) of SKG mice in 1, 2 and 3 weeks after mannan treatment are shown (n = 6). **D,** Representative histology of SKG mice 3 weeks after mannan treatment (H&E staining). Bars; 200 μm. **E,** Cells infiltrating into the synovial tissues were counted (n = 6). Data are shown as mean ± SEM. * = $P < 0.05$.

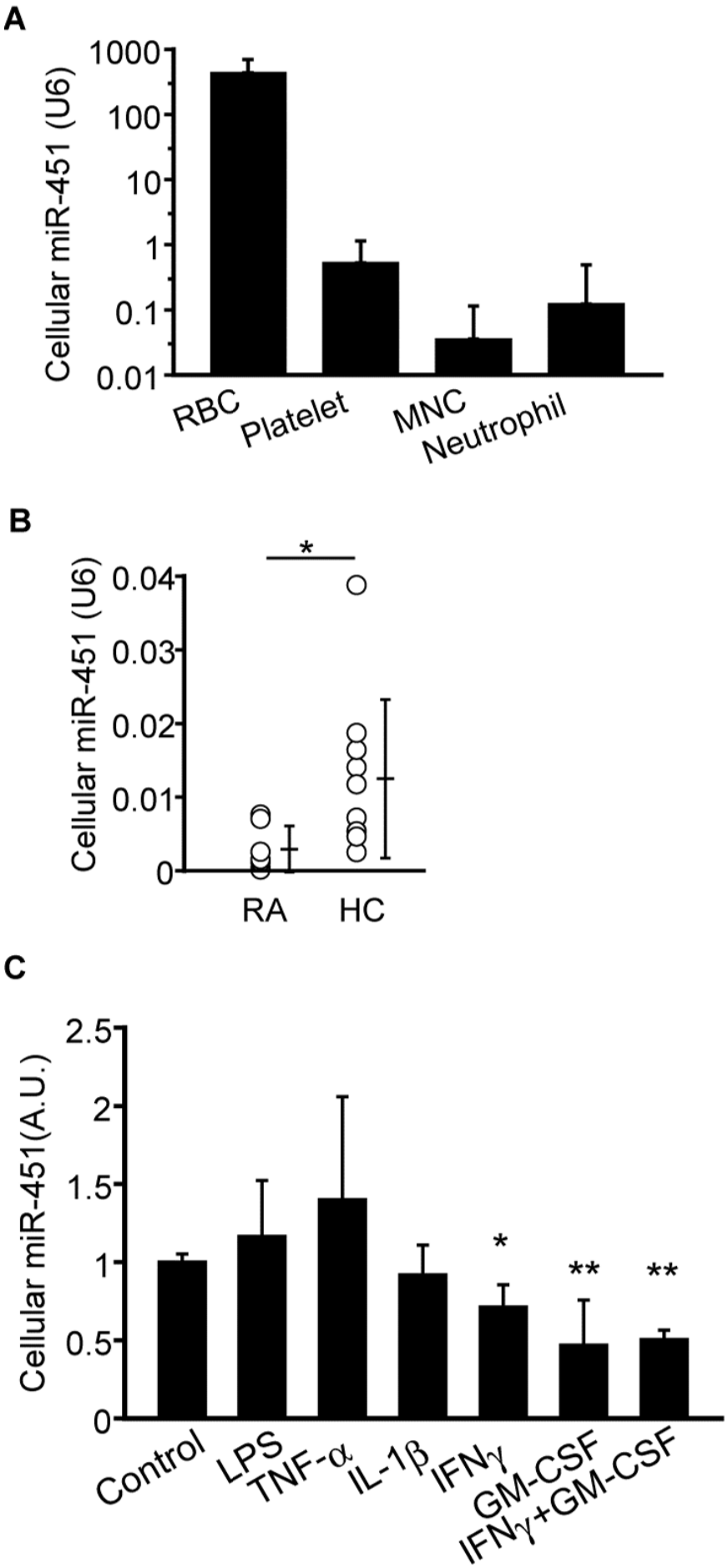


Figure 1.

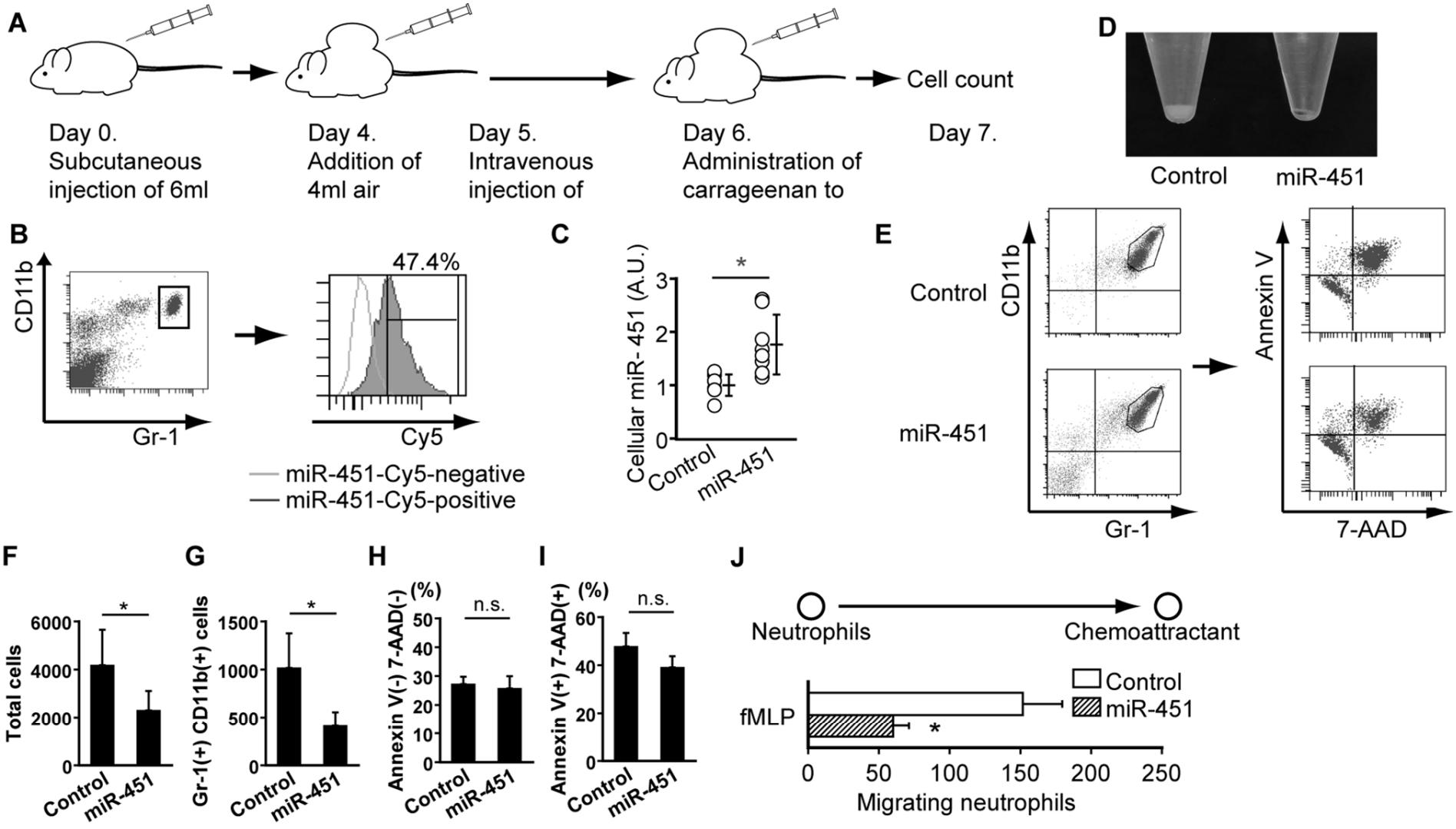

A
Day 0.
Subcutaneous
injection of 6ml
Day 4.
Addition of
4ml air
Day 5.
Intravenous
injection of
Day 6.
Administration of
carrageenan to
Cell count
Day 7.
D
Control
miR-451
B
CD11b
Gr-1
47.4%
Cy5
miR-451-Cy5-negative
miR-451-Cy5-positive
C
Cellular miR-451 (A.U.)
Control
miR-451
E
Control
miR-451
CD11b
Gr-1
Annexin V
7-AAD
F
Total cells
Control
miR-451
G
Gr-1(+) CD11b(+) cells
Control
miR-451
H
Annexin V(-) 7-AAD(-) (%)
n.s.
Control
miR-451
I
Annexin V(+) 7-AAD(+) (%)
n.s.
Control
miR-451
J
Neutrophils
Chemoattractant
Control
miR-451
fMLP
Migrating neutrophils

Figure 2.

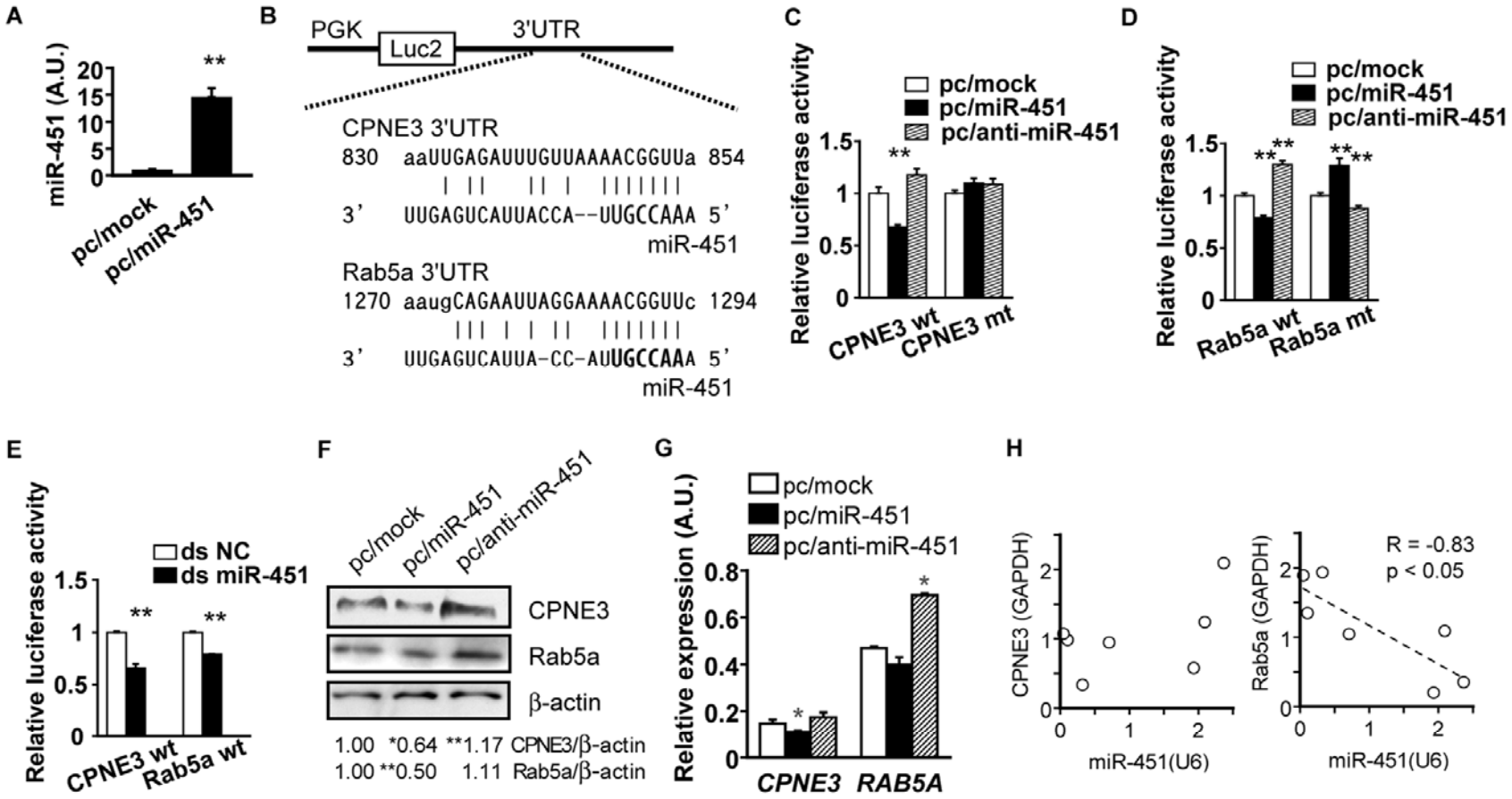
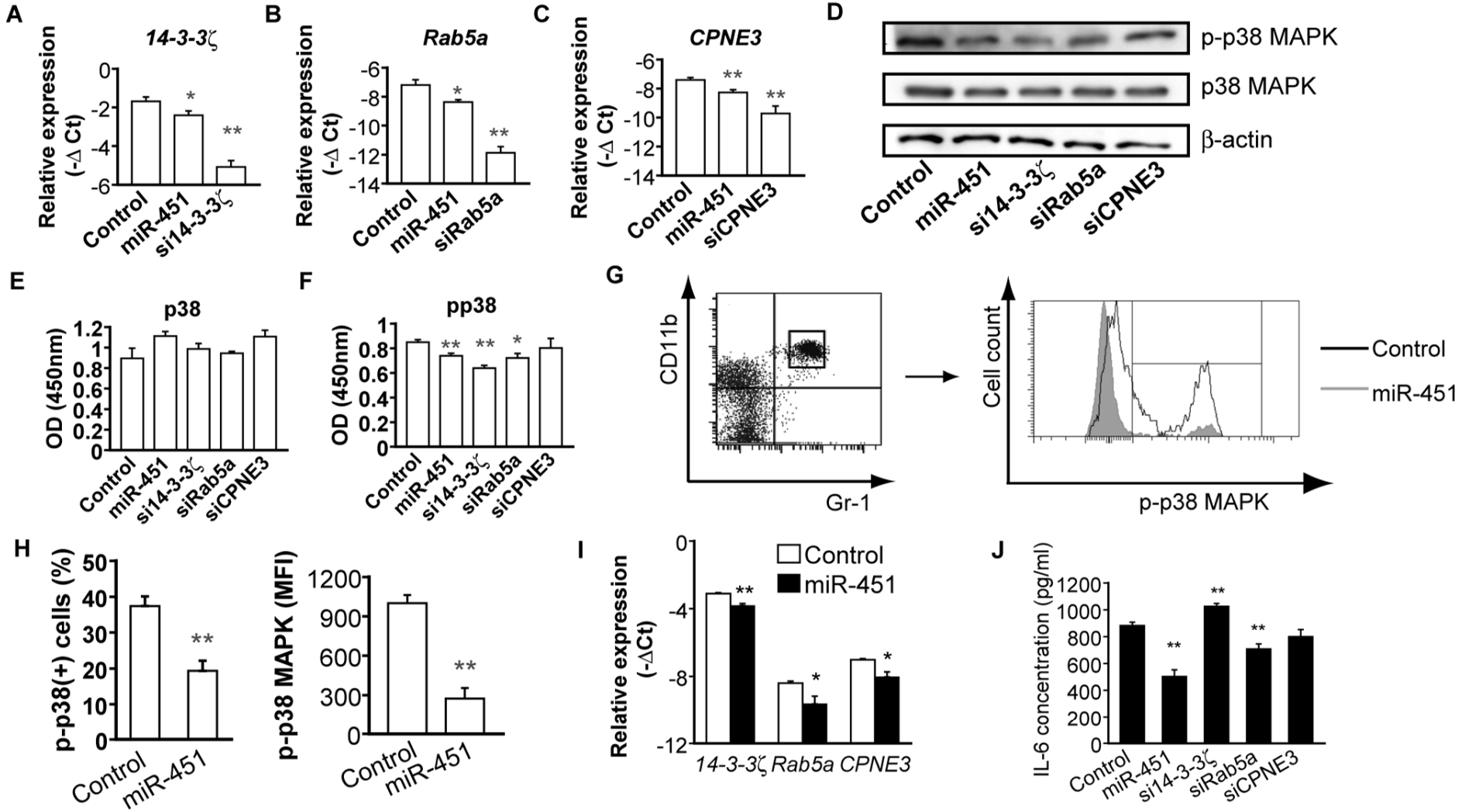

A
miR-451 (A.U.)
20
15
10
5
0
**
pc/mock
pc/miR-451
B
PGK
Luc2
3'UTR
CPNE3 3'UTR
830 aaUUGAGAUUUGUUAAAACGGUUa 854
3' UUGAGUCAUUACCA--UUGCCAAA 5'
miR-451
Rab5a 3'UTR
1270 aaugCAGAAUUAGGAAAACGGUUc 1294
3' UUGAGUCAUUA-CC-AUUGCCAAA 5'
miR-451
C
Relative luciferase activity
pc/mock
pc/miR-451
pc/anti-miR-451
1.5
1
0.5
0
**
CPNE3 wt
CPNE3 mt
D
Relative luciferase activity
pc/mock
pc/miR-451
pc/anti-miR-451
1.5
1
0.5
0
**
**
**
**
Rab5a wt
Rab5a mt
E
Relative luciferase activity
ds NC
ds miR-451
1.5
1
0.5
0
**
**
CPNE3 wt
Rab5a wt
F
pc/mock
pc/miR-451
pc/anti-miR-451
CPNE3
Rab5a
β-actin
1.00 *0.64 **1.17 CPNE3/β-actin
1.00 **0.50 1.11 Rab5a/β-actin
G
Relative expression (A.U.)
pc/mock
pc/miR-451
pc/anti-miR-451
0.8
0.6
0.4
0.2
0
*
*
CPNE3
RAB5A
H
CPNE3 (GAPDH)
2
1
0
0
1
2
miR-451(U6)
R = -0.83
p < 0.05
Rab5a (GAPDH)
2
1
0
0
1
2
miR-451(U6)

Figure 3.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14-3-3ζ
Relative expression (-Δ Ct)
Control
miR-451
si14-3-3ζ
B
Rab5a
Relative expression (-Δ Ct)
Control
miR-451
siRab5a
C
CPNE3
Relative expression (-Δ Ct)
Control
miR-451
siCPNE3
D
p-p38 MAPK
p38 MAPK
β-actin
Control
miR-451
si14-3-3ζ
siRab5a
siCPNE3
E
p38
OD (450nm)
Control
miR-451
si14-3-3ζ
siRab5a
siCPNE3
F
pp38
OD (450nm)
Control
miR-451
si14-3-3ζ
siRab5a
siCPNE3
G
CD11b
Gr-1
Cell count
p-p38 MAPK
Control
miR-451
H
p-p38(+) cells (%)
Control
miR-451
p-p38 MAPK (MFI)
Control
miR-451
I
Relative expression (-ΔCt)
Control
miR-451
14-3-3ζ Rab5a CPNE3
J
IL-6 concentration (pg/ml)
Control
miR-451
si14-3-3ζ
siRab5a
siCPNE3

Figure 4.

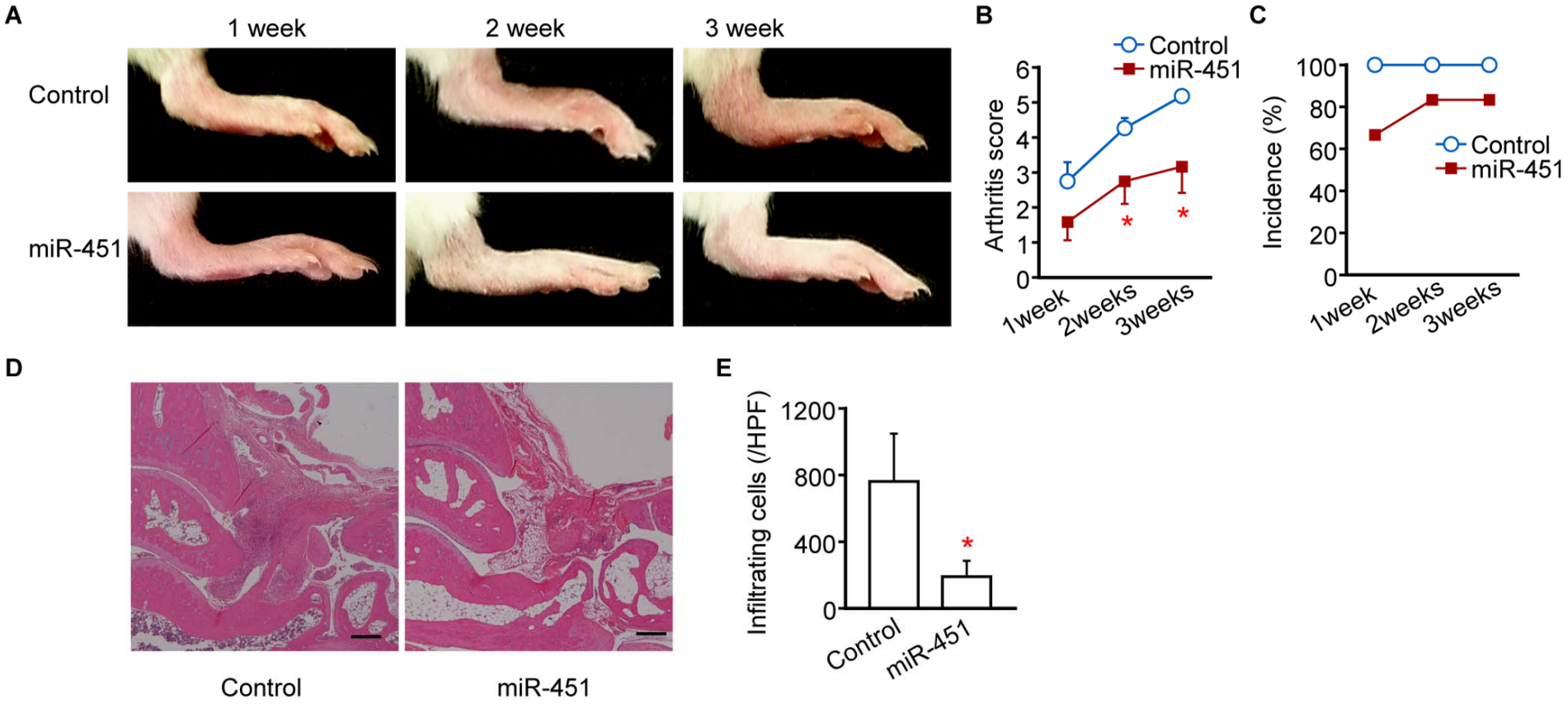
A
1 week
2 week
3 week
Control
miR-451
B
Control
miR-451
Arthritis score
0
1
2
3
4
5
6
1week
2weeks
3weeks
*
*
C
Incidence (%)
0
20
40
60
80
100
Control
miR-451
1week
2weeks
3weeks
D
Control
miR-451
E
Infiltrating cells (/HPF)
0
400
800
1200
*
Control
miR-451

Figure 5.